

ORIGINAL RESEARCH

Downregulation of TSPAN1 inhibits angiogenesis in triple-negative breast cancer cells by regulating the PI3K/Akt signaling pathway

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Abstract

Background: Triple-negative breast cancer (TNBC) progression and invasion highly depends on abnormal angiogenesis. While tetraspanin 1 (TSPAN1) has been implicated in the malignant progression of TNBC, its relationship in tumor angiogenesis remains poorly understood. **Methods:** Tumor samples were collected from patients with TNBC and analyzed using clinical pathological and Western blot analyses. TSPAN1 expression was knocked down in the TNBC cell line, Hs-578T using siRNA technology. The angiogenic ability of human umbilical vein endothelial cells (HUVECs) was evaluated by lumen formation assay. The Phosphatidylinositol 3-Kinase (PI3K)/Protein Kinase B (Akt) signaling pathway was detected using western blots. **Results:** TSPAN1 overexpression in TNBC tumors is linked to metastasis and PI3K/Akt pathway activation. Knockdown of TSPAN1 in TNBC cells, significantly suppressed the tube formation ability of co-cultured HUVECs. Mechanistically, TSPAN1 knockdown significantly inhibited the activity of the PI3K/Akt pathway. **Conclusions:** In conclusion, TSPAN1 promotes TNBC angiogenesis via activation of PI3K/Akt signaling pathway.

Keywords

Triple-negative breast cancer; TSPAN1; PI3K/Akt; Angiogenesis

1. Introduction

Breast cancer is the most common disease in women worldwide, with a distinct subtype, triple-negative breast cancer (TNBC), with a high metastasis index and proliferation rate [1, 2]. Even though breast cancer diagnostics and therapeutics have advanced significantly, TNBC poses a high risk of angiogenesis, metastasis, and drug resistance due to lack of particular targets and focused medicines [3]. Angiogenesis has a crucial part in the metastatic process. Tumor cells can spread to other locations by using new blood vessels as a means of leaving the original tumor and establishing colonies at distant sites [4, 5]. New therapeutic approaches for TNBC require a deeper comprehension of the molecular processes controlling angiogenesis and metastasis.

Tetraspanin 1 (TSPAN1) belongs to the class of tiny transmembrane proteins known as the tetraspanin family. Studies demonstrate that TSPAN regulates the malignant features of cancer cells, such as invasion, metastasis, apoptosis, and proliferation [6]. TSPAN1 is an oncogene that plays important role in tumor growth by increasing its expression in a variety of human malignancies [7–9]. Previous research revealed that TSPAN1 promotes the proliferation and spread of breast cancer cells [10]. Additionally, *TSPAN1* gene expression is linked to advanced gastric cancer and may play a role in angiogenesis

and the epithelial-mesenchymal transition [11]. Furthermore, TSPAN1 has been found to enhance breast cancer cell proliferation and metastasis through the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway [10]. It also promotes epithelial-mesenchymal transition and facilitates metastasis of bile duct carcinoma via PI3K/Akt signaling [12].

The signaling pathway between PI3K and Akt is essential for tumor development, neovascularization, and cell proliferation [13]. Previous research discovered that PI3K/Akt/Mammalian Target of Rapamycin (mTOR) signaling is critical and this signaling pathway is dysregulated in nearly all cases of advanced TNBC [14]. The purpose of this study was to look at TSPAN1's involvement and mechanism in regulating tumor angiogenesis in TNBC.

2. Methods

2.1 Cell lines

Human umbilical vein endothelial cells (HUVECs) and TNBC Hs-578T cells were obtained from the American Type Culture Collection (ATCC). These cells were cultivated at 37 °C in presence of 5% carbon dioxide (CO₂) and humidity in Dulbecco's modified Eagle's medium (DMEM, MT10013CV, Gibco, Waltham, MA, USA) supplemented with

3.7 g/L sodium bicarbonate, 10% fetal bovine serum (FBS, FB12999102, Gibco, Waltham, MA, USA), and 50 units/mL of penicillin and streptomycin (15-140-163, Invitrogen, Carlsbad, CA, USA).

2.2 Cell transfection

TSPAN1 expression in Hs-578T cells was inhibited by specific siRNA targeting TSPAN1 (Thermo Fisher Scientific, Waltham, MA, USA). Scrambled siRNA-transfected cells (si-NC, Thermo Fisher Scientific, Waltham, MA, USA) served as controls. The sequences of the TSPAN1 si-1 group were 5'-GGCUCACGACCAAAAAGUAtt-3' (sense) and 5'-UACUUUUUGGUCGUGAGCCtt-3' (antisense). The sequences of the TSPAN1 si-2 group were: 5'-GCUUUUGUAUGACAUCGAtt-3' (sense), 5'-UCGGAUGUCAUACAAAAGCtg-3' (antisense). The sequences of the scrambled siRNA group were 5'-UUCUCCGAACGUGUCACGUDtT-3' (sense), 5'-ACGUGACACGUUCGGAGAAAdTt-3' (antisense). Lipofectamine 3000 (L3000008, Thermo Fisher Scientific, Waltham, MA, USA) was used to transfect Hs-578T cells with 50 nM siRNA following manufacturer's instructions. TSPAN1 expression was detected 48 h after transfection.

2.3 Angiogenesis assays

For this assay, 50 μ L Matrigel (8 mg/mL, CB-40234C, Thermo Fisher Scientific, Waltham, MA, USA) was added into the lower chamber of a 24-well Transwell and incubated at 37 °C for 1 h. Following this, 1×10^4 HUVECs and Hs-578T cells were co-cultured at 37 °C for 24 h in the lower and upper chambers of Transwell, respectively. Images of tubular structures were captured using an optical microscope (Inverted Fluorescence Microscope, Olympus Corporation, Tokyo, Japan), and the images were counted using ImageJ software (v2021.8.0).

2.4 Western blot analysis

Total protein was extracted from tissues and cells using Radioimmunoprecipitation Assay (RIPA) buffer (P0013B, Beyotime, Shanghai, China), and the concentration was measured using a Bicinchoninic Acid Assay (BCA) kit (P0009, Beyotime, Shanghai, China). Isolated proteins were split into various pieces based on their molecular weights using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The samples were then transferred to a polyvinylidene fluoride membrane (PVDF, Millipore, Billerica, MA, USA), blocked with 5% skim milk for 1 h at 25 °C, and incubated with the corresponding primary antibody overnight at 4 °C. After that, the membrane was treated for 1 h with a secondary antibody coupled with horseradish peroxidase (P2369, Beyotime, Shanghai, China). Ultimately, an enhanced chemiluminescence detection kit (P10010, NCM Biotech, Shanghai, China) was used to visualize the protein band. The bands were quantified using ImageJ 8.0 software, with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference protein. The primary antibodies (1:1000, Abcam, Cambridge, UK) analysed were as follows: TSPAN1 (ab254730), PI3K (ab302958), p-PI3K (ab278545), Akt (ab314110), p-Akt (ab8805), Cluster

of Differentiation 31 (CD31, ab281583), Matrix Metalloproteinase (MMP-2, ab92536), MMP-9 (ab76003) and GAPDH (ab32096).

2.5 Tumor samples

Paraffin-embedded specimens from 126 patients with triple-negative breast cancer admitted to Pingshan District People's Hospital between January 2021 and December 2023 were gathered for retrospective investigation. Patient's age at diagnosis, their tumor grade, tumor size, clinical stage, and lymph node status were obtained from the medical data record. The Tumor-Node-Metastasis (TNM) method served as the basis for the histological type. Prior to the follow-up deadline, all patients received routine follow-ups by phone or in-person visits. Informed consent was obtained from those patients in their follow-up visit and their tissue specimens were taken for further investigation. All these cancer tissues were tested for the expression of TSPAN1 by immunohistochemistry.

2.6 Inclusion criteria

Confirmed diagnosis: all patients included in the study were diagnosed with TNBC by pathological examination. Specifically, their tumor showed no expression of the estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER2).

Sample integrity: the patient's tumor tissue and corresponding clinical data (including age, tumor size, tumor grade, clinical staging, and lymph node status, *etc.*) were complete and available.

Ethical approval: all patients or their legal representatives signed an informed consent form, agreeing that their anonymized clinical information will be used for research purposes. The hospital's ethics committee approved the research plan (approval number: 2023013).

2.7 Exclusion criteria

Non-primary tumor: patients diagnosed with metastatic breast cancer or non-primary breast cancer confirmed by pathological evaluation were excluded.

Concurrent malignancies: patients diagnosed with other malignant tumors before or at the time as TNBC diagnosis were excluded.

Patients receiving neoadjuvant therapy: patients who had received chemotherapy, radiation therapy, or other systemic treatments before surgery were excluded, as these treatments could affect the biological characteristics of the tumor.

Incomplete clinical data: patients with missing or incomplete clinical data were excluded to ensure the accuracy and reproducibility of research results.

2.8 Statistical analyses

The statistical analysis was carried out using IBM SPSS software (version 25, Armonk, NY, USA). The relationship between TSPAN1 expression levels and clinicopathological characteristics was investigated using the chi-square test or Fisher's exact probability method. Differences between the two groups were examined using two-tailed Student's *t*-test. Tukey's *post*

hoc test was employed to determine significance in the group differences. A *p* value of less than 0.05 was considered statistically significant.

3. Results

3.1 TSPAN1/PI3K/Akt signaling is upregulated in triple-negative breast cancer

To study the significance of TSPAN1 in breast cancer, we first reviewed data from a public database, The Cancer Genome Atlas (TCGA), and showed that TSPAN1 expression was much greater in breast cancer tissues than in normal breast tissues (Fig. 1A) ($p < 0.05$). We then tested cancer and adjacent tissues from 20 TNBC patients and found that the expression of TSPAN1 and CD31 was higher in TNBC tissues, accompanied by PI3K/Akt signaling pathway activation (Fig. 1B) ($p < 0.001$). These results suggested that high expression of TSPAN1, activation of PI3K/Akt signaling pathway, and angiogenesis are closely related to the development of TNBC.

We further examined the association between TSPAN1 expression in TNBC tumor samples and clinical pathological features of breast cancer, such as age, tumor size, T classification, N classification, and TNM stage. Table 1 illustrates the approximate correlation between TSPAN1 expression in TNBC and lymph node metastases, N classification, and T classification. These findings showed that TSPAN1 might play a major role in TNBC metastasis.

3.2 Knockdown of TSPAN1 inhibits angiogenesis in breast cancer cells

To elucidate the role and mechanism of TSPAN1 in the development of TNBC, we performed siRNA-mediated knockdown of TSPAN1 in the TNBC cell line Hs-578T (Fig. 2A) ($p <$

0.001). The results showed that knockdown of TSPAN1 could inhibit the expression of matrix metalloproteinase (MMP)-2 and MMP-9 angiogenic factors (Fig. 2A) ($p < 0.001$). Additionally, HUVECs angiogenesis assay showed that knockdown of TSPAN1 could reduce the number of blood vessels (Fig. 2B) ($p < 0.001$). The above results showed that knockdown of TSPAN1 inhibits angiogenesis by reducing MMP-2 and MMP-9.

3.3 Knockdown of TSPAN1 inhibits the PI3K/Akt pathway

Because previous results showed elevation of TSPAN1 and increased activation of PI3K/Akt signaling pathway in TNBC tissues, we next investigated whether TSPAN1 regulates the PI3K/Akt pathway in TNBC cells. Knocking down TSPAN1 affects the PI3K/Akt signaling pathway (Fig. 3) ($p < 0.001$), leading to decreased angiogenesis in TNBC. These findings imply that TSPAN1 plays an essential role in TNBC angiogenesis via the PI3K/Akt signaling pathway.

4. Discussion

Numerous physiological and pathological processes, such as cancer, the immune system, fertility, and infectious diseases, have been linked to TSPAN1. In the context of tumorigenesis, TSPAN1 may stimulate tumor growth through its effects on angiogenesis, immune function, platelet coagulation, and infection [15]. The association between TSPAN1 expression and TNBC was carefully assessed in this study. TSPAN1 was shown to be strongly expressed in TNBC, its expression was connected to the clinicopathological features and metastasis in patients with TNBC. Additionally, TSPAN1 was shown to suppress the angiogenic potential of HUVECs in TNBC cells. These findings revealed that TSPAN1 may be involved

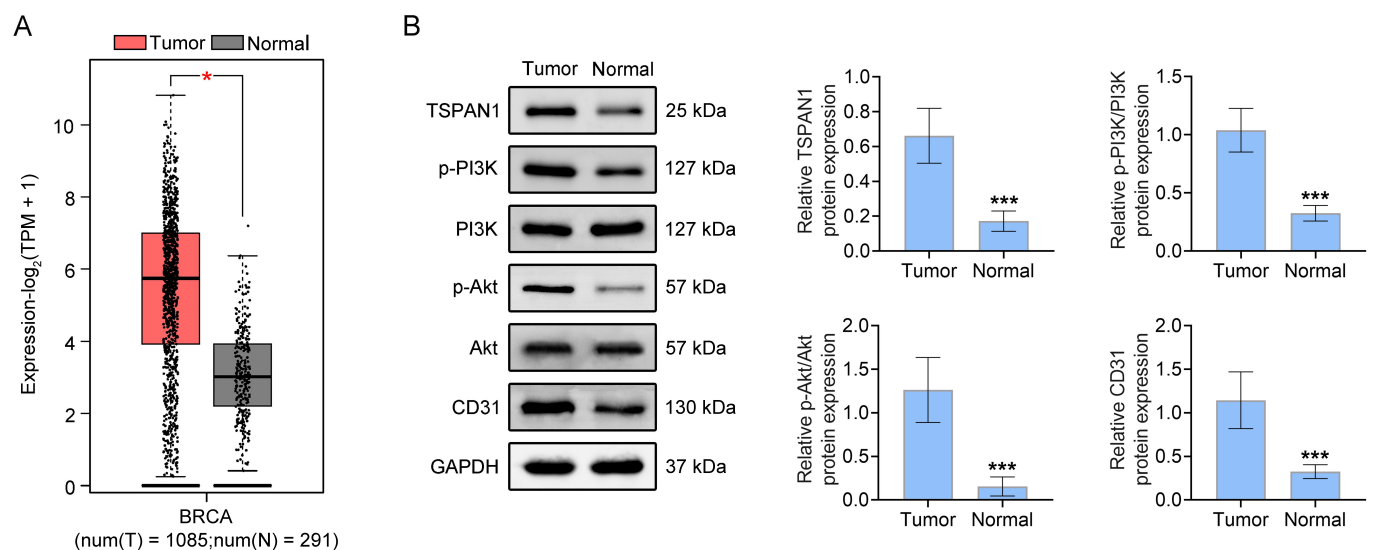


FIGURE 1. TSPAN1/PI3K/Akt signaling is upregulated in triple-negative breast cancer. (A) GEPIA website analysis of the expression difference of TSPAN1 in breast cancer tissues and normal tissues. (B) Protein expression of TSPAN1, PI3K, p-PI3K, Akt, p-Akt and CD31 in TNBC cancer tissue and adjacent tissue. *: $p < 0.05$; ***: $p < 0.001$. $n = 20$. PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; TSPAN1: tetraspanin 1; TPM: Transcripts Per Million; BRCA: Breast invasive carcinoma; CD: Cluster of Differentiation; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

TABLE 1. Association of expression of TSPAN1 with pathological categories in BRCA tumor samples.

Pathological category	Total	TSPAN1 express		χ^2	p
		Low	High		
Age (yr)					
≥42	68	19	49	0.144	0.704
<42	58	18	40		
Tumor size (cm)					
≥3	62	8	54	15.947	<0.001
<3	64	29	35		
T category					
T1	20	12	8	11.494	0.003
T2	97	24	73		
T3	9	1	8		
N category					
N0	62	21	41	1.398	0.497
N1	45	12	33		
N2	19	4	15		
TNM stage					
I	13	10	3	16.236	<0.001
II	91	23	68		
IIIA	22	4	18		

p < 0.05 was considered significant. *n* = 126. T: Tumor; N: Node; TSPAN1: tetraspanin 1; TNM: Tumor-Node-Metastasis.

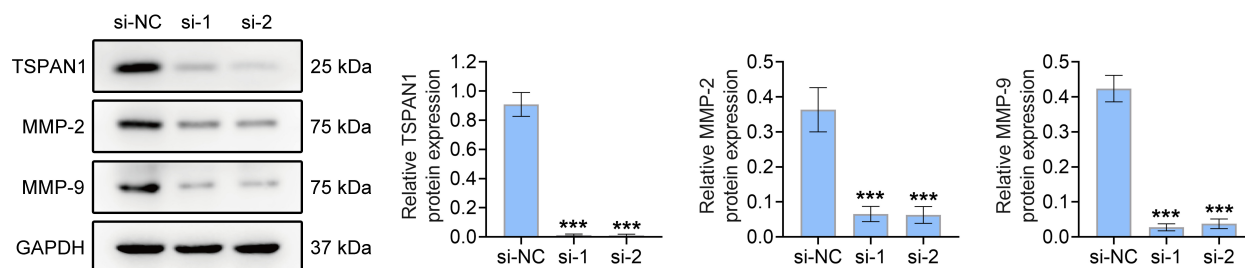
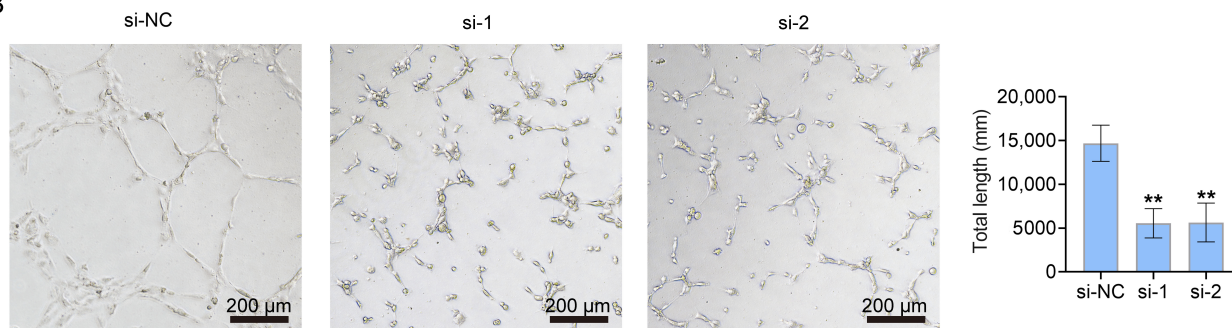
A**B**

FIGURE 2. Knockdown of TSPAN1 inhibits angiogenesis in breast cancer cells. (A) Western blotting to detect the expression of TSPAN1, MMP-2, MMP-9. (B) Detection of angiogenesis ability of HUVECs. Values are presented as mean $\pm$ SD. ***p* < 0.01 vs. si-NC group; ****p* < 0.001 vs. si-NC group. *n* = 3. si-NC: siRNA-transfected cells; TSPAN: tetraspanin; MMP: Matrix Metalloproteinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

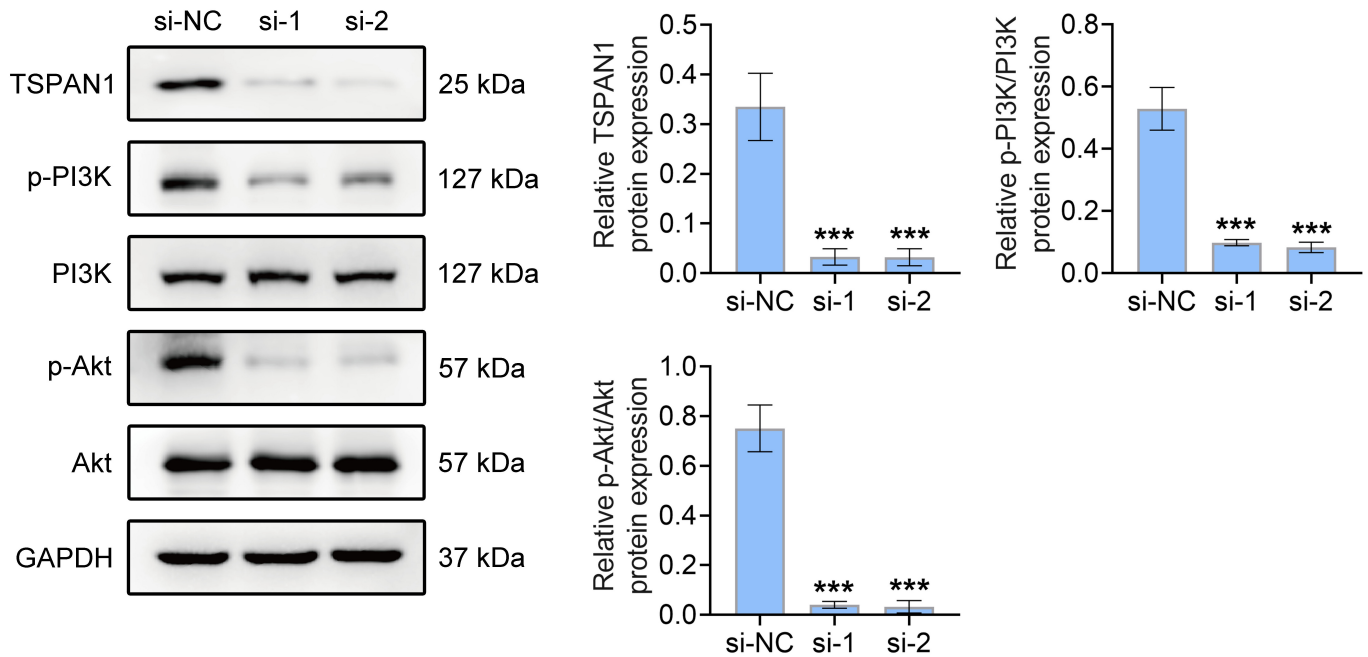


FIGURE 3. Knockdown of TSPAN1 inhibits the PI3K/Akt pathway. Western blotting to detect the expression of TSPAN1, PI3K, p-PI3K, Akt, p-Akt in TNBC Hs-578T cells. Values are presented as mean $\pm$ SD. *** $p < 0.001$ vs. si-NC group. $n = 3$. TSPAN: tetraspanin; PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; si-NC: siRNA-transfected cells; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

in TNBC angiogenesis by controlling the PI3K/Akt signaling pathway. These findings point to TSPAN1 as a viable therapeutic target for treating TNBC.

Angiogenesis, adhesion, degradation, migration, and reattachment are all components of tumor metastasis. A variety of adhesion molecules, proteolytic enzymes, and cytokines participate in these processes [16]. Studies have demonstrated that TSPAN1 expression is positively linked with tumor angiogenesis [11]. Tumor cells can release a range of angiogenic molecules like vascular endothelial growth factor (VEGF) that can directly cause angiogenesis in endothelial cells by stimulating the production of new blood arteries from preexisting ones, which in turn can activate endothelial cells [17]. Additionally, MMPs—primarily MMP-9 and MMP-2—secreted by endothelial cells contribute to the invasive and morphogenic processes that occur during angiogenesis [18]. MMP-2 and MMP-9 promote angiogenesis by degrading basement membrane and extracellular matrix, providing space for endothelial cell migration and invasion. Furthermore, MMP-9 can release VEGF by degrading matrix proteins, enhancing angiogenesis [19]. Our study showed that knockdown of TSPAN1 in TNBC cells could inhibit angiogenesis by suppressing the expression of MMP-2 and MMP-9.

Studies have indicated a strong association between angiogenesis and the activation of PI3K/Akt signaling pathway. In addition to regulating angiogenic factors, such as angiopoietin and carbon monoxide, activation of PI3K/Akt signaling pathway has been observed to increase the synthesis of VEGF [20]. The PI3K/Akt signaling pathway can also directly activate matrix metalloproteinases such as MMP-2 and MMP-9. Because of this activation, MMP can break down the extracellular matrix, creating channels and room

for endothelial cell migration and the angiogenesis process, thus creating new blood vessels [21]. Our findings revealed that TSPAN1 knockdown might decrease the activity of the PI3K/Akt pathway in TNBC cells, consequently decreasing angiogenesis.

The high expression of TSPAN1 in TNBC and its critical involvement in tumor angiogenesis and activation of PI3K/Akt signaling pathway suggest that TSPAN1 is a highly promising therapeutic target. Targeting TSPAN1, through gene silencing, small molecule inhibitors or antibody drugs, is expected to provide more effective treatment options for TNBC patients. Future studies are needed to further verify the safety and efficacy of TSPAN1-targeted therapies. Future studies should also focus on clinical application and optimizing combination treatment strategies to promote the clinical application of TSPAN1 targeted therapy.

This study also has some limitations. The present research on TSPAN1 and PI3K/Akt signaling pathways in triple-negative breast cancer relies solely on *in vitro* models, which may not fully simulate the complex physiological and pathological environment in the human body. Future studies are needed to better investigate the specific mechanism of action of TSPAN1 in tumor cells and confirm *in vivo* angiogenesis in xenograft models, combining multi-target treatment strategies to improve the therapeutic effect.

5. Conclusions

Our findings show that TSPAN1 expression is substantially associated with TNBC metastasis, suggesting that it could be a therapeutic target for anti-metastatic TNBC treatment. Additionally, knockdown of TSPAN1 inhibits the angiogenesis of

TNBC by inhibiting MMP-2 and MMP-9 expression, as well as the PI3K/Akt signaling pathway.

AVAILABILITY OF DATA AND MATERIALS

The data analyzed in this study were derived from the medical records of 126 patients with triple-negative breast cancer admitted to Pingshan District People's Hospital between January 2021 and December 2023. The use of these data was approved by the Ethics Committee of Pingshan District People's Hospital (Approval no. 2023013). The data are available for review upon reasonable request, subject to patient confidentiality and ethical considerations.

AUTHOR CONTRIBUTIONS

YGW—designed the study and carried them out. YGW, WXC, JZ—supervised the data collection; interpreted the data; prepared the manuscript for publication and reviewed the draft of the manuscript. YGW, WXC—analyzed the data. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was obtained from the Ethics Committee of Pingshan District People's Hospital (Approval no. 2023013). Written informed consent was obtained from a legally authorized representative for anonymized patient information to be published in this article.

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Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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